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## AGGREGATION OF PENAEID SHRIMP LARVAE DUE TO MICROBIAL EPIBIONTS

D.H. LEWIS, J.K. LEONG\* and C. MOCK\*

*Department of Veterinary Microbiology and Parasitology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843 (U.S.A.)*

*\*National Marine Fisheries Service, NOAA, Southeast Fisheries Center, Galveston Laboratory, 4700 Avenue U, Galveston, TX 77550 (U.S.A.)*

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### ABSTRACT

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*Pseudomonas piscicida*, *Aeromonas formicans* and *Flavobacteria* sp. were involved in aggregation of hatchery reared *Penaeus stylirostris* larvae. Aggregation was experimentally reproduced with pure cultures of these organisms at densities of  $10^4$  cells per ml. Adding at least 3  $\mu\text{g/ml}$  gentamycin, 10  $\mu\text{g/ml}$  nalidixic acid, 0.1  $\mu\text{g/ml}$  acridine or Cutrine Plus®\* into test suspensions prevented aggregation of the shrimp larvae.

### INTRODUCTION

Microbial fouling sometimes becomes a major limiting factor in the production of certain commercially important decapod crustaceans. Although all developmental stages of decapod may be afflicted, the condition is most serious in egg and larval stages. Gross characteristics of the condition are the setaceous appearance of eggs or developing larvae followed by death of affected animals. Both filamentous and non-filamentous agents have been incriminated in deaths associated with microbial fouling; but agents that have been most often diagnosed are the filamentous bacterium, *Leucothrix mucor*, and the epicommensal protozoan, *Zoothamnium* sp. (Johnson, 1974). These agents apparently use the surface of the animal only as a substrate and rely on dissolved nutrients in the surrounding water for nourishment. Their role in disease would thus appear to be a passive one involving either mechanical entanglement of developmental stages by filamentous agents or occlusion of

\*The use of trade names does not imply endorsement by the authors or institutions which they represent.

respiratory surfaces by non-filamentous agents (Fisher, 1977).

Microbial fouling was suspected following frequent, extensive losses of *Penaeus stylirostris* zoeal and mysid larvae at the hatchery of the National Marine Fisheries Service Southeast Fisheries Center's Biological Laboratory, Galveston, TX, during the spring and summer of 1980. Aggregation of the developing shrimp was a consistent feature associated with the deaths. Before clumping, food particles and other particulate matter became entrapped in the setae of the larvae and the animals could only swim with great difficulty. Informal communications with workers in the United States and elsewhere suggested that the aggregation phenomenon might be a widespread problem of hatchery reared shrimp. Filamentous agents appear not to be involved in deaths associated with the phenomenon, and aggregation of the larvae has generally been a prelude to complete decimation of the developing shrimp stocks. The present study was thus initiated to better understand the phenomenon.

## MATERIALS AND METHODS

Larvae and water samples were collected from the hatchery on five occasions and transported to the Department of Veterinary Microbiology and Parasitology, Texas A&M University, from March to July 1980. The March, May and June samples were collected at a time when hatchery larvae demonstrated signs characteristic of the clumping phenomenon, which ultimately resulted in complete loss of the developing shrimp. Samples collected during April and July were collected at a time when hatchery losses were minimal.

Samples were processed within 6 h of collection. Ammonia, pH and salinity measurements were made on all water samples. In addition, water samples were diluted ten-fold in Marine Broth (Difco, Inc., Detroit, MI) and the bacterial concentration estimated by extinction dilution turbidity after incubation at 48 h 20°C. Ten-, 1- and 0.1 ml volumes of water samples were placed in 100 ml sterile aged seawater and filtered on 0.45 µm nitrocellulose membrane (Schliecher & Schuell, Inc., Keene, NH). The saturated membranes were placed on marine agar for a qualitative assessment of the water microflora. After 48 h incubation at 20°C, plates were examined under a dissecting microscope with oblique light and various colony types were transferred to marine broth for further study and identification (Lewis, 1973).

The larvae from each collection were subdivided into three 50-ml aliquots, each containing approximately 40 larvae per ml. One of the aliquots was set aside for periodic gross and microscopic observation, while two of the aliquots were centrifuged  $800 \times g$  10 min, washed three times in filter sterilized aged seawater and resuspended in 1 ml of sterile aged seawater. Bacteria were isolated from the suspension by streaking on marine agar and Leucothrix medium (Kelly and Brock, 1969).

Three bacterial isolates identified as *Pseudomonas piscicida*, *Aeromonas formicans* and *Flavobacterium* sp. were derived from water and larval samples

in which the clumping phenomenon had been observed. Also, one isolate identified as a *Vibrio* sp. was found in all water and larval samples and was not believed to be associated with clumping. These bacterial isolates were used in the experiments designed to study the aggregation phenomenon. The organisms were grown 30 h at 20°C in marine broth after which time a saturated culture containing  $2 \times 10^9$  organisms per ml had developed. The cells were concentrated by centrifugation at  $8000 \times g$  for 20 min at 6°C, washed two times and reconstituted to one-half the original volume in filter sterilized aged seawater. Shrimp larvae demonstrating no obvious abnormalities were collected on 69 mm mesh plankton net, washed with 4 l sterile aged seawater five times to remove most of the food and other adsorbed detritus, and subdivided into 24 200-ml aliquots each containing approximately 1000 larvae. Washed bacterial cells were added to three replicate aliquots at concentrations of  $10^2$  to  $10^7$  bacteria per ml. Six control aliquots received no washed bacterial cells. All aliquots were aerated and the larvae examined for aggregation after a 48-h incubation period at 20°C.

Drug testing was conducted in 200 ml aged seawater containing approximately 5 larvae per ml and  $10^4$  *A. formicans*, *Flavobacterium* sp. or *Ps. piscicida* cells per ml in replicates of three at each drug concentration. The larval preparations were incubated 4 h before receiving the various drugs.

The antibiotics, nalidixic acid (Neg Gram® Winthrop Laboratories, 90 Park Avenue, New York), and gentamycin (Garamycin®, Schering Corp., Galloping Hill Road, Kenilworth, NJ) were selected for the study on the basis of susceptibility patterns of the three isolates incriminated in the aggregation phenomenon. Antibiotics were diluted to yield 0.5, 1.0, 3.0, 5.0, 10, 20 and 50 µg antibiotic per ml in the test solutions. Acriflavin (2, 8 diaminoacridine) and Cutrine-plus® (Crescent Research Chemicals, Inc., Paradise Valley, AZ) were incorporated in the study on the basis of their reported antibacterial activity (Hoffman and Meyer, 1974). These compounds were added to the test suspensions to yield 0.01, 0.05, 0.1, 0.2 and 0.3 µg active acriflavin or copper per ml. All aliquots were aerated and the larvae examined for aggregation after a 48 h incubation period at 20°C.

## RESULTS

Ammonia levels of hatchery water samples were relatively low (0.5 ppm or less) and pH levels ranged from 8.2 to 8.4. Holding salinities ranged from 28 to 31‰.

Filamentous bacteria were not recovered from larvae or water samples used in this study. However, sessile protozoa, *Zoothamnium* sp., were observed on the larval samples collected during June, 48 h after collection. The protozoa were not observed during initial examination. At the time of collection, a ciliate, tentatively identified as *Anophrys* sp., was observed within the body fluids of some of the larvae collected during March.

The concentrations of bacteria in water samples wherein aggregation was

observed was approximately the same as those in which the phenomenon was not observed. However, qualitative differences between the two types of samples were reflected by the presence of *Ps. piscicida*, *A. formicans* or *Flavobacterium* sp. in both water and larval samples wherein clumping was observed (Table I).

TABLE I

Bacterial analysis of samples of shrimp larvae and their culture water collected March–July 1980

		<i>Achr. ichthyodermis</i>	<i>A. formicans</i>	<i>Aerococcus</i> sp.	<i>Flavobacterium</i> sp.	<i>Pediococcus homeri</i>	<i>Ps. diminuta</i>	<i>Ps. enalia</i>	<i>Ps. piscicida</i>	<i>Ps. tetraeolens</i>	<i>Vibrio</i> sp.	No. bacteria per ml water	
March	Water	X	X		X			X	X		X	10 <sup>3</sup>	Aggregation observed
	Larva		X	X	X	X	X		X		X		
	Larva	X	X	X	X	X	X		X		X		
April	Water			X			X			X	X	10 <sup>3</sup>	Normal larvae
	Larva	X		X		X	X			X	X		
	Larva					X				X	X		
May	Water		X		X			X	X		X	10 <sup>4</sup>	Aggregation observed
	Larva	X	X		X		X		X		X		
	Larva	X	X		X		X		X		X		
June	Water		X		X			X	X		X	10 <sup>3</sup>	Aggregation observed
	Larva		X		X				X		X		
	Larva		X		X				X		X		
July	Water	X						X		X	X	10 <sup>4</sup>	Normal larvae
	Larva	X						X		X	X		
	Larva	X								X	X		

Aggregation of the larvae was observed after 24 h incubation in suspensions that received sufficient bacteria to yield at least 10<sup>4</sup> *Ps. piscicida* or *Flavobacterium* sp. cells per ml. The majority of the aggregates contained viable larvae. Larvae exposed to 10<sup>5</sup> or greater *A. formicans* cells per ml died within 10 h. Aggregation was not observed in the larvae exposed to either *A. formicans* or *Vibrio* sp. Larvae exposed to 10<sup>6</sup> or greater *Ps. piscicida*, *A. formicans*, *Flavobacterium* sp. or *Vibrio* sp. cells per ml died within 24 h of exposure (Table II).

Aggregation did not occur in those suspensions containing at least 3 µg/ml gentamycin, 10 µg/ml nalidixic acid, 0.1 µg/ml acriflavin or Cutrine Plus. Neither of the antibiotics appeared to be toxic to the shrimp larvae when in-

TABLE II

Response of shrimp larvae (5 per ml) incubated at various bacterial concentrations.  
Aggregation not observed among six control groups of larvae

Bacteria	Concentration (cells per ml)					
	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>	10 <sup>7</sup>
<i>Ps. piscicida</i>	0	0	A	AD	D	D
	0	A	A	AD	D	D
	0	0	A	A	D	D
<i>A. formicans</i>	0	0	0	D	D	D
	0	0	0	D	D	D
	0	0	D	D	D	D
<i>Flavobacterium</i> sp	0	0	A	A	D	D
	0	0	A	A	D	D
	0	0	A	A	D	D
<i>Vibrio</i> sp.	0	0	0	D	D	D
	0	0	0	0	D	D
	0	0	0	0	D	D

Control larval aggregation not observed in six replicates.

A = Aggregation of larvae.

D = Death of larvae.

AD = Aggregation preceded death of larvae.

0 = No apparent effect on larvae prior to 48 h exposure.

incorporated into maintenance water at 50 µg/ml or less; however, acriflavin caused death in the larvae within 6 h at concentrations ≥ 0.2 µg/ml, Cutrine Plus caused death of larvae within 6 h at concentrations ≥ 0.3 µg/ml (Table III). Larvae in suspensions containing washed *A. formicans* cells died within 24 h of exposure in all preparations except the one receiving the highest concentration of gentamycin (50 µg/ml).

DISCUSSION

Microbial epibionts play an important role in the well-being of many marine crustaceans. The appendages of these animals are often populated with high concentrations of bacteria that are periodically cropped and serve as a food source for the host as well as for other animals involved in the grooming procedure (Anderson and Stephens, 1969; Bauer, 1979). In other instances, microbial epibionts multiply over the surface of the egg membranes or larval gill membranes, either directly occluding respiratory surfaces or setting the stage for growth of filamentous bacteria or sessile protozoa and ultimate death of the host. The larger, more easily visible organisms are thus often incriminated as principal agents when, in fact, their role in the infestation may be secondary.

TABLE III

Data for trials testing efficacy of various drugs to control aggregation

Chemical/Bacteria	Drug concentration (μg active ingredient/ml) three replicates per concentration <sup>a</sup>						
	0.01	0.05	0.1	0.2	0.3	0.5	1.0
Acriflavin							
<i>Ps. piscicida</i>	A	A,A,0	0	0,D,D	D	D	D
<i>Flavobacterium</i> sp.	A	A,0,0	0	0,D,D	D	D	D
<i>A. formicans</i>	D	D	D	D	D	D	D
Cutrine plus							
<i>Ps. piscicida</i>	A	A	0	A,D,0	D	D	D
<i>Flavobacterium</i> sp.	A	A	0	D	D	D	D
<i>A. formicans</i>	D	D	D	D	D	D	D
	0.5	1.0	3.0	5.0	10	20	50
Gentamycin							
<i>Ps. piscicida</i>	A	A,0,0	0	0	0	0	0
<i>Flavobacterium</i> sp.	A	A,0,0	0,0	0	0	0	0
<i>A. formicans</i>	D	D	D	D	D	D	0
Nalidixic acid							
<i>Ps. piscicida</i>	A	A	A,A,0	0	0	0	0
<i>Flavobacterium</i> sp.	A	A	A,A,0	A,0,0	0	0	0
<i>A. formicans</i>	D	D	D	D	D	D	D

A = Aggregation of larvae.  
D = Death of larvae.  
0 = Aggregation not observed.  
<sup>a</sup>Effect observed in all three replicates except where otherwise indicated.

The present episode illustrates aggregation as another consequence of epi-biotic fouling. Under experimental conditions, relatively high numbers of bacteria (at least 10<sup>4</sup> organisms per ml) were required to cause aggregation. Water quality parameters did not appear unusual, thus factors which allowed the ubiquitous organisms involved in the episode to become established and bring about the aggregation phenomenon remain unknown. Nonetheless, anti-biotics and other drugs, which diminish the viability and/or activity of the organisms even when the organisms are present at relatively high levels, ap-parently minimize aggregation.

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